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Ferritin levels in microglia depend upon activation: Modulation by reactive oxygen species

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Abstract

Iron is one of the trace elements playing a key role in the normal cellular metabolism. Since an excess of free iron is catalyzing the Fenton reaction, most of the intracellular iron is sequestered in the iron storage protein ferritin. The binding of iron into ferritin is well described for physiological conditions, however, under certain pathophysiological situations, the efficiency of this process is unknown. In the brain, microglial cells are among others the cell population most importantly responsible for the maintenance of the extracellular environment. These cells might undergo activation, and little is known about the expression of ferritin during activation of microglial cells. Therefore, we tested the microglial model cell line RAW264.7 for the expression of ferritin after LPS activation. A significant decrease in the levels of the ferritin H-chain during activation and a significant increase in the early recovery phase were found. We were able to demonstrate that reactive oxygen species are responsible for a suppression of the H-chain of ferritin, whereas iNOS expression and NO synthesis are counteracting the reactive oxygen species effect. The balance of reactive oxygen species and NO production are, therefore, determining expression levels of the ferritin H-chain during activation of microglial cells.

Keywords: Microglia; Activation; Ferritin; ROS; iNOS

1. Introduction

In the brain, the content of ferritin is high and can be strongly altered during aging or in specific regions in brains of Alzheimer's and Parkinson's patients [1–3]. Besides oligodendrocytes, microglia appears to have a high ferritin content, which mostly consists of L-chains [4,5]. This is also a characteristic of organs storing iron, like liver and spleen. Thus, microglia plays an important role in brain iron homeostasis [6,7]. Another characteristic feature of these cells is their ability to produce reactive oxygen and nitrogen species upon activation [8–12].

Interestingly, the activation of microglia is accompanied by a reversible oxidation of their own protein pool due to activation [13,14]. This condition might be dangerous for the cell itself and neighbouring neurons, since some oxidants, like superoxide radicals, H_2O_2 and nitric oxide, are able to diffuse in biological tissue for some distance. On the other hand, such oxidizing species are altering the iron sequestering function of ferritin [15,16] and furthermore trigger the release of iron directly from ferritin in vitro [17–22].

Due to the fact that microglial activation produce vast amounts of reactive oxygen and nitrogen species and in consequence damage their own protein pool, it is important to know how activation of microglial cells is influencing the ferritin content in these cells and which role oxidizing species are playing in the modulation of ferritin expression. Therefore, we utilized in this study mouse RAW264.7 cells as a known microglial model and examined their ability to maintain a ferritin steady-state concentration in response to LPS activation.

2. Materials and methods

2.1. Cell culture

RAW264.7 macrophages were cultured in RPMI medium supplemented with 10% foetal bovine serum (Biochrom). Cells were plated at a density of

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$2–3 \times 10^6$ in T75 flacons and grown for 2 days. For harvesting, cells were scraped in medium and centrifuged for 8 min at 4 °C and 200×*g* followed by two steps washing with PBS.

2.2. RAW cell stimulation and treatment with the different chemical compounds

For the iron depletion experiment cells were incubated 16 h with 100 μM Desferioxamine. RAW cells were activated by treatment with 10 μg/ml LPS for 16 h. Then either the cells were immediately harvested or further cultivated with normal RPMI medium for the next 24 h. In the case of iNOS inhibition, the potent inhibitors 1400 W hydrochloride (Calbiochem) and L-NIL (Calbiochem) were added to the cells during LPS activation at 25 μM of each. To analyze the effect of free radicals cells were additionally incubated with *N*-tert-butyl-α-phenylnitrone (PBN, Sigma), a commonly used free-radical spin trap, at a concentration of 600 μM.

2.3. Immunoblot analysis

Briefly, proteins were extracted from RAW cells by gentle shaking in lysis buffer (1 mM DTT in 25 mM Tris pH 7.4 and 50 mM NaCl) 1 h on ice, followed by centrifugation at 14,000×*g* (Beckman) for 30 min at 4 °C. The supernatant was removed for protein determination by the BCA kit (Pierce). Identical amounts of total protein were boiled in loading buffer before electrophoretic separation (12% SDS-PAGE), transferred to a PVDF membrane, blocked with 5% skim milk in PBS/0.5% Tween 20, incubated with primary (anti-human-ferritin, Dako) and secondary antibody (anti-rabbit-IgG HRP-conjugated, Sigma), and finally analyzed for immunoreactivity using a POD chemiluminescence kit (NEN). Pre-stained precision markers were used to estimate the apparent molecular weight of the protein bands. The relative optical densities were quantified using TabLab software.

2.4. Detection of nitrite/nitrate species (NO_x)

From a stock nitrite standard (0.1 M sodium nitrite) a fresh 0.1 mM sodium nitrite solution was prepared immediately followed by 6 serial 2-fold dilutions to generate the nitrite standard reference curve. 50 μl of the nitrite standards and the cell culture supernatants were pipette into a 96-well plate. Accordingly, 100 μl Griess (Fluka) reagent was added and incubated for 10–15 min at room temperature. The formed purple/magenta colour was detected by measuring the absorption at 550 nm in a plate reader.

2.5. ELISA for detection of protein oxidation

The protein carbonyl content was taken as a measure for protein oxidation. Protein carbonyls were determined in cell lysates (4 mg/ml in lysis buffer with 1 mM BHT) by an ELISA as introduced by Buss et al. [23] with modifications described by Sitte et al. [24]. Primary anti-dinitrophenyl–rabbit–IgG antiserum (Sigma) and a secondary monoclonal anti-rabbit–POD-conjugated IgG (Sigma) were used as detection system. Development was performed with *o*-phenylene diamine and H_2O_2.

2.6. Statistical evaluation

Data are presented as mean±S.D. Significance of differences was tested using the Student's test considering $P<0.05$ as significantly different.

3. Results

3.1. Reduction of Ferritin by iron depletion

Ferritin is the most important intracellular iron storage, and the cellular ferritin level is regulated by the effective iron concentration in the cell. To test whether the ferritin level in RAW264.7 is regulated as usual, we compared the ferritin level in RAW264.7 cells under normal conditions with conditions of cellular iron depletion, simulated by adding 100 μM desferrioxamine, known as iron chelator (Fig. 1A). The antibody is recognizing only the H-chain of ferritin as reported before [16]. The ferritin H-chain level was strongly reduced after desferrioxamine treatment approximately by 70% (Fig. 1B). This proves the direct relationship between cellular free iron concentration and cellular ferritin level.

3.2. LPS stimulated ferritin reduction and ferritin overload during recovering in RAW264.7 cells

Our primary goal was the investigation of the ferritin level during activation of RAW264.7 cells. Therefore, we stimulated RAW cells using LPS. As shown in Fig. 1C, after 16-h incubation with LPS, the level of the ferritin H-chain is minimally, but significantly decreased by about 20%. However,

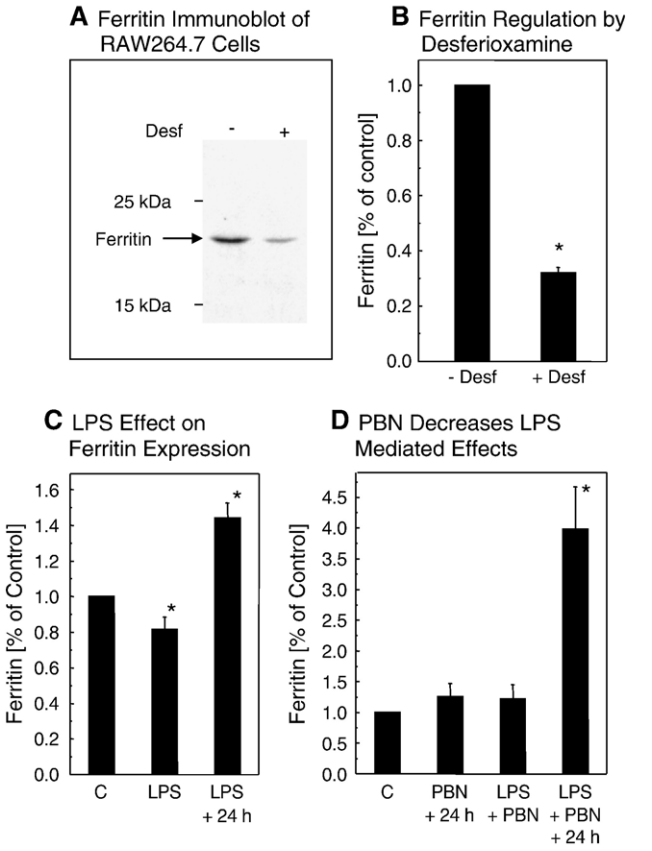

Fig. 1. Regulation of H-chain ferritin levels in RAW264.7 cells during activation. Cells were cultivated and treated with the iron chelating agent desferioxamine as described in the Materials and methods section. In panel A, an immunoblot developed with an anti-ferritin antibody of cell lysate of RAW264.7 cells with and without desferioxamine (Desf) incubation is shown. Panel B demonstrates the quantitative evaluation of 3 independent experiments with the control (−Desf) set as 1 (mean±S.D., *$P<0.05$). The effect of LPS was tested after a 16-h incubation with LPS (LPS) and following 24 h of recovery (LPS+24 h). Panel C demonstrates the quantitative evaluation of 4 independent experiments with the control (C) set as 1 (mean±S.D., *$P<0.05$). The effect of the antioxidant PBN on the expression of H-chain of ferritin was tested in non-activated and activated Raw264.7 cells. Panel D demonstrates the quantitative evaluation of 3 independent experiments with the control (C) set as 1 (mean±S.D., *$P<0.05$).

another 24 h later (recovering phase without LPS), the H-chain of ferritin rebounded and achieved an approximately 40% higher level as compared to the control level. Since we demonstrated earlier that reactive oxygen species induce a reduction of the ferritin level in RAW264.7 cells [16] and LPS, as some other activators, is inducing an oxidative burst in RAW264.7 cells [13,25] we assumed, the reduction of ferritin might be triggered by oxidants produced during the activation of RAW cells. The high level of ferritin following recovering might display an adaptive response to oxidative stress, since it has been shown that ferritin appears to be a protein with antioxidative properties.

3.3. Co-incubation with the free radical scavenger PBN prevents LPS stimulated ferritin reduction

To test the idea whether the presence of reactive oxygen species might be responsible for the decreased ferritin level in LPS-activated microglia, RAW264.7 cells were stimulated for 16 h with LPS in the presence of PBN, a well-known free radical scavenger. As demonstrated in Fig. 1D, the co-treatment LPS/PBN could prevent the LPS stimulated reduction of the ferritin H-chain and resulted in a ferritin H-chain level higher as compared to the untreated control. This is showing the involvement of ROS in reducing the H-chain ferritin level in LPS-activated macrophages/microglia. Interestingly, after a 24-h recovery phase of the RAW264.7 cells the H-chain ferritin level was increased to about 400% in the presence of PBN. This indicates an improved adaptive response of microglial cells protected by PBN. On the other hand, PBN alone without any stimulation did not affect the level of the H-chain of ferritin (Fig. 1D).

To test whether the addition of PBN was really effective in preventing protein modification in activated microglial cells, we measured the protein carbonyl content as a marker of protein modification. As demonstrated in Fig. 2, the amount of protein carbonyls is extremely elevated to more then 250% in the LPS stimulated RAW64.7 cells as compared to the untreated control cells. Whereas addition of PBN during LPS activation revealed a lesser formation of protein carbonyls, about a third lower than in cells treated with LPS alone.

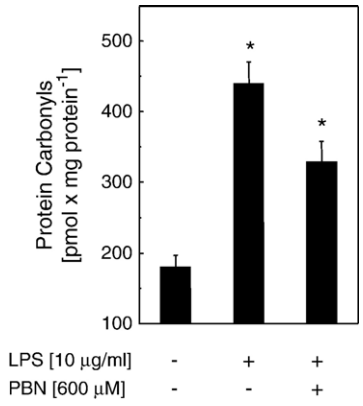

Fig. 2. PBN modulates the protein oxidation in LPS-treated RAw264.7 cells. Protein oxidation in RAW264.7 cells was determined 16 h after LPS or LPS and PBN addition by measuring protein carbonyl levels as described in the Materials and methods section. The data represent the mean±S.D. of 3 independent experiments with 4 measurements each (*$P<0.05$).

3.4. LPS stimulated ferritin reduction is boosted by simultaneous iNOS inhibitor treatment

It is well known that reactive microglial cells activated by LPS are releasing besides reactive oxygen species also reactive nitrogen species. This is the consequence of the iNOS induction and the production of the free radical nitric oxide (NO). NO has been shown to bind to iron sulphur centres of enzymes, thus interfering with the functioning of those enzymes such as cytosolic aconitase also known as iron regulatory protein IRP1 [26,27]. On the other hand, NO has been proposed to act as a factor preventing the effects of reactive oxygen species by a very efficient reaction with the superoxide radical forming peroxynitrite, a potent oxidant, but unable to diffuse larger distances in biological tissues [28–31].

Therefore, we tested the role of reactive nitrogen species on the ferritin production in activated RAW264.7 cells. To investigate the influence of NO production on ferritin levels, RAW cells were simultaneously incubated with specific iNOS inhibitors 1400 W and L-NIL during their LPS activation. The addition of iNOS inhibitors caused an additional ferritin reduction around 20–25% (Fig. 3A). This would agree with the fact that NO is reducing the reactive oxygen level, and therefore, the inhibition of NO production is further reducing the content of the ferritin H-chain due to enhanced ROS action.

In order to be sure, that the LPS activation was successful in our hands, NO_x concentrations were determined by using Griess reagent showing a strong production of NO_x by LPS, almost completely abolished by simultaneous addition of iNOS inhibitors (Fig. 3B).

4. Discussion

On one hand, ROS production is a natural result of the oxidative metabolism in mammalian cells connected with oxidation of proteins and consequently with increased intracellular proteolysis [16,32]. On the other hand, this basal ROS production is boosted by activated microglia/macrophages as defence mechanism against several pathogens. Iron plays a critical role during this process due to its catalyzing effect on the generation of free radicals by hydrogen peroxide via Fenton reaction. Under physiological conditions, excess intracellular iron is sequestered in ferritin, in order to prevent this process. Ferritin levels are modulated by cellular iron content [33–36]. We were able to demonstrate also the modulation of ferritin H-chain expression by free iron in RAW264.7 cells (Fig. 1). However, the ferritin content is decreased in presence of reactive oxygen species, due to the oxidation of intracellular ferritin and consequent proteasomal degradation and a reduced ferritin synthesis [16]. Such an oxidative stress-mediated effect on the ferritin H-chain was confirmed by our results demonstrated here with LPS-activated RAW cells showing a

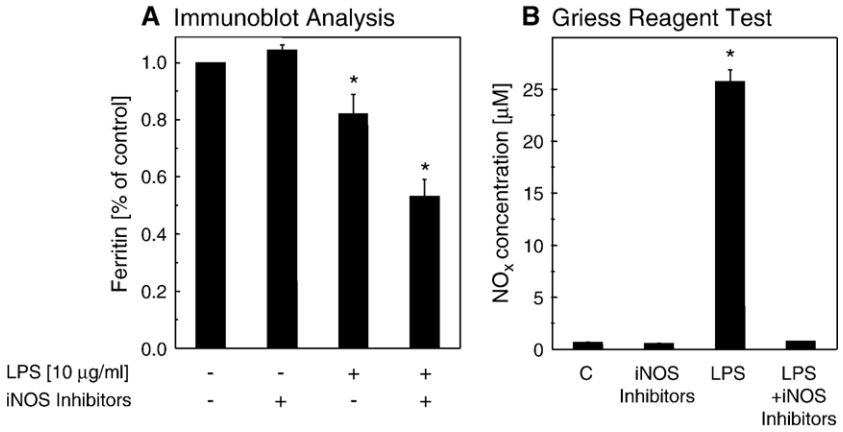

Fig. 3. iNOS inhibition facilitates LPS-mediated effects on ferritin levels. Ferritin expression was measured either without addition, 16 h after LPS addition or the addition of LPS and iNOS inhibitors. As iNOS inhibitors 1400W and L-NIL were used in parallel. Panel A shows the quantitative evaluation of the ferritin expression determined by immunoblots (mean±S.D., $n=4$, $^*P<0.05$). Panel B shows the formation of NO_x after treatment with LPS, or LPS and iNOS inhibitors (mean±S.D., $n=5$, $^*P<0.05$).

decreased ferritin level under continuous LPS stimulation which is inhibitable by PBN treatment.

Previous work of us [24,37–40] and others [41–43] demonstrated the role of the proteasomal system in the removal of oxidized proteins from the intracellular protein pool. A self-oxidation of intracellular proteins during microglial activation and the efficiency of the proteasome in the restitution of the intracellular protein pool were already demonstrated [13,16,32]. These results could be confirmed by measurements of increased protein carbonyl formation due to activation of RAW264.7 cells and the partial reduction by the usage of PBN (Fig. 2).

LPS-mediated activation of microglial cells is going along with the production of multiple factors and reactive species. One of most potent changes during microglial activation is the high elevated NO production caused by induction of iNOS [44–48]. LPS stimulation also causes high fluxes of superoxide radical induced by NADPH oxidase activation [25]. Under such conditions, Mn-superoxide dismutase is induced in microglial cells [49]. However, the produced NO is giving a number of chemical reactions, one of the most important is the reaction with the superoxide anion radical and the formation of peroxynitrite [28–31]. This reaction is taking place at a nearly diffusion limited rate and was already several-fold demonstrated to take place in activated macrophages and microglia [28,50]. At least these four reactive oxygen species: superoxide anion radical, NO, $ONOO^-$ and H_2O_2 influence directly or indirectly the ferritin level and the binding activity of iron regulatory proteins (IRPs) [15,16,26,27,51–57]. Besides the protein synthesis modulated by the IRPs, the ferritin level is of course also regulated by the proteolysis rate.

As does iron chelation by desferal, endogenous NO production abolishes completely aconitase activity by disassembling the [Fe-S] cluster and strongly activates IRE binding by IRP1 resulting in inhibited ferritin synthesis over a long time period [26,27,51]. In contrast, under reducing conditions, the full IRP1/IRE-binding capacity is 50% reduced, caused by a 50% decrease in IRP1 protein level [52]. A reduction is also caused by $ONOO^-$-mediated IRP1 tyrosin nitration in response to endogenous NO synthesis [51]. However, also H_2O_2 [15,16,26,27,53,54] and superoxide anion radical [55–57] inactivate the c-aconitases and support induction of IRP/IRE binding and inhibition of ferritin synthesis, which appears to be a fast response (maximal IRP1 activation between 30 and 60 min) [26,27,53]. Therefore, we speculate that the main part of ferritin reduction in LPS stimulated RAW cells is due to inhibition of ferritin synthesis via increased IRP1/IRE binding induced by ROS, since PBN prevents this effect.

It has been demonstrated in vitro that different oxidants as superoxide radical, NO, $ONOO^-$ and H_2O_2 increases the proteolytical susceptibility of ferritin [21]. These data were confirmed in H_2O_2 treated RAW macrophages, where significant increased ferritin degradation was observed [16]. Additionally, it has to be mentioned that in vitro different oxidants, as for example superoxide radical and H_2O_2, are able to release iron from ferritin, which can catalyze the decomposition of hydroperoxides resulting in new toxic radicals [18,21,22]. Thus, ferritin can be oxidatively damaged and in consequence is degraded by proteasome [16,21]. Therefore, LPS induced ROS production in macrophages might accelerate ferritin proteolysis. At this point, it should be mentioned that at moderate H_2O_2 concentration H-ferritin seems to be faster degraded as compared to L-ferritin, despite its less susceptibility for oxidation [16], possibly in order to prevent additional hydrogen peroxide production by the H-ferritin-mediated ferroxidase activity [58–60]. The above discussed facts concerning inhibited ferritin synthesis and accelerate ferritin proteolysis are explanations for the observed decreased protein level of ferritin after LPS stimulation in macrophages and the preventing effect by PBN.

Interestingly, NO seems to have a protecting effect on ferritin, since inhibition of NO formation by adding iNOS inhibitors reveals a much stronger ferritin reduction in LPS stimulated RAW cells. Although the result of the NO reaction with superoxide is the very potent oxidant peroxynitrite, it seems to be likely that the enhanced formation of NO might reduce the oxidative damage to cells [61–63]. This is likely due

to the short diffusion distance of the formed peroxynitrite in biological tissue and the prevention of ROS-mediated effects and signalling in dependency of SOD activity [31]. Otherwise, RNS are suggested to work as scavenger for peroxide radicals produced through the Fenton reaction. Under LPS stimulation reactive nitrogen species (RNS) appear to have an ROS effect eliminating function [64]. In our model, the inhibition of iNOS is enhancing the ROS-mediated depletion of ferritin, indicating a protective role of the produced NO on the ferritin pool.

Taken together, all these data indicate that intracellular ferritin level is strongly regulated by complex interaction between iron load, cytokine release (for example TNF-α) and NO and superoxide radical production in activated microglial cells. Such findings implicate that the iron-dependent free radical production may represent another useful defence system, where NO has a major role in regulation of its strength.

Whether the ROS are directly affecting on the ferritin molecule or the mobile iron pool, or the IRP1 and IRP2 molecules remains to be investigated in microglial cells. However, independent on the mechanism the results presented in this study identify a reduction of the most important cellular iron-binding protein ferritin in consequence of ROS production in LPS-activated RAW macrophages.

Acknowledgement

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